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Site-Selective Functionalization of (sp³)C–H Bonds Catalyzed by Artificial Metalloenzymes Containing an Iridium-Porphyrin Cofactor

Yang Gu, Sean N. Natoli, Zhennan Liu, Douglas S. Clark, and John F. Hartwig*

Abstract: The selective functionalization of one C–H bond over others in nearly identical steric and electronic environments can facilitate the construction of complex molecules. We report site-selective functionalizations of C–H bonds, differentiated solely by remote substituents, catalyzed by artificial metalloenzymes (ArMs) that are generated from the combination of an evolvable P450 scaffold and an iridium-porphyrin cofactor. The generated systems catalyze the insertion of carbenes into the C–H bonds of a range of phthalan derivatives containing substituents that render the two methylene positions in each phthalan inequivalent. These reactions occur with site-selectivity ratios of up to 17.8:1 and, in most cases, with pairs of enzyme mutants that preferentially form each of the two constitutional isomers. This study demonstrates the potential of abiotic reactions catalyzed by metalloenzymes to functionalize C–H bonds with site selectivity that is difficult to achieve with small-molecule catalysts.

Introduction

C–H bond functionalization has changed the logic of chemical synthesis by enabling the direct addition of functional groups to a wide range of available starting materials.^[1] Small-molecule transition-metal catalysts can lead to site-selective C–H bond functionalization,^[2] but many of the strategies for achieving selectivity rely on localized steric effects, directing groups, or both. Thus it is challenging for small-molecule catalysts to distinguish between C–H bonds with similar local steric and electronic properties rendered inequivalent by the lack of symmetry of a molecule. However, enzymes, such as cytochromes P450 (CYPs), catalyze the oxidation of C–H bonds that are nearly equivalent with high site selectivity.^[3] Such reactions often occur within the

biosynthetic pathways of complex natural products, such as terpenes, alkaloids, or steroids, all of which contain an abundance of similar C–H bonds.^[4]

Although they can react with high selectivity, natural enzymes install a limited range of functional groups during biosynthesis. One strategy to expand the scope of reactions catalyzed by enzymes and improve the efficiency by which these reactions can occur is to conduct directed evolution. Directed evolution has proven to be a powerful methodology that allows chemists to extend the fitness of biocatalysts toward unnatural reactions.^[5] Through directed evolution, in which many rounds of iterative mutagenesis are conducted, hemoproteins have been repurposed to catalyze a range of unnatural group transfer reactions to C–C multiple bonds and C–H bonds with high activity and selectivity.^[6]

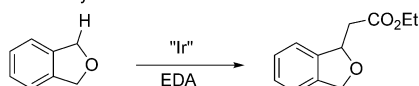
A complementary strategy to expand the scope of known abiological transformations is to construct artificial metalloenzymes (ArMs). These systems incorporate unnatural cofactors into evolvable protein scaffolds,^[7] and the resulting ArMs are capable of catalyzing abiological reactions, often with high selectivity. Several ArMs have been constructed by exploiting the biotin-streptavidin interaction,^[8] unnatural amino acid anchoring,^[9] and formal replacement of the native heme cofactor with synthetic porphyrin cofactors containing noble metals.^[10]

Our laboratory reported the first example of enzymatic carbene insertions into (sp³) C–H bonds, including intermolecular functionalizations. These reactions were catalyzed by artificial metalloenzymes containing an Ir(Me)-MPIX cofactor,^[11] and such reactivity provides a platform to evaluate whether Ir(Me)-containing ArMs could be evolved to catalyze site-selective reactions of C–H bonds that are nearly equivalent and are known to yield a mixture of products with small-molecule catalysts.^[12] Phthalan derivatives were chosen as model substrates because they have two sites that contain (sp³) C–H bonds with similar steric and electronic environments because unsubstituted phthalan undergoes reaction with carbene precursors catalyzed by a P450 containing the Ir(Me)-MPIX cofactor (Scheme 1).^[11]

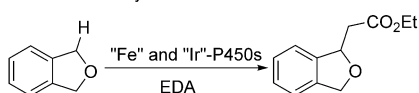
Here, we report that 4-substituted phthalans react to give products that are distinguished only by the position of a remote substituent and react with pairs of Ir(Me)-containing CYP119 mutants to give one of the two opposite constitutional isomers as the major product for most of the derivatives. We also show that the reactions occur with high chemoselectivity for C–H bonds over typically reactive functional groups, such as N–H bonds, and in select cases with high enantioselectivity.

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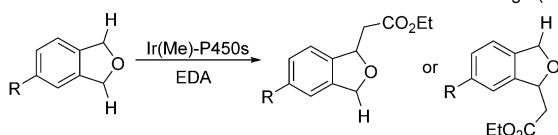
A. small-molecule Catalysts for intermolecular C-H Functionalization



B. Laboratory Evolved Biocatalysts for Enantioselective C-H Functionalization



C. This work: site- and Chemo-selective C-H Functionalization using Ir(Me) P450s



Scheme 1. Intermolecular insertion of acceptor-only carbenes into (sp^3)C–H bonds with small-molecule catalysts and biocatalysts.

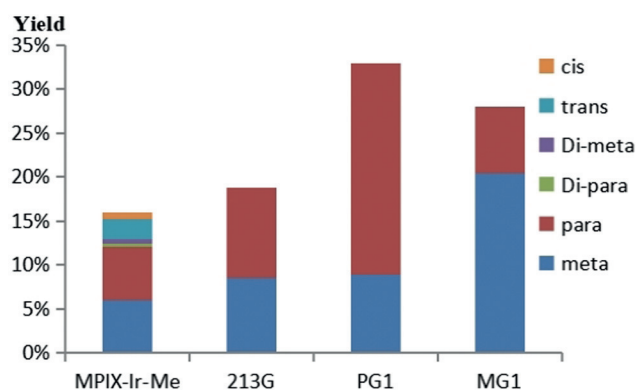
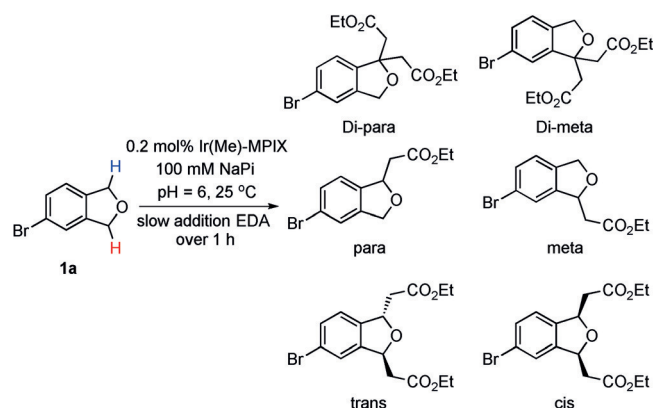
Results and Discussion

Reactivity of the Free Ir(Me)-MPIX Cofactor and Engineered Ir(Me)-Hemoproteins

To determine the role of the protein scaffold in controlling site selectivity, we first assessed the reactivity and site selectivity of the free Ir(Me)-MPIX cofactor for the insertion of the carbene from ethyl 2-diazoacetate (EDA) into the C–H bond of a 4-substituted phthalan. To do so, we studied the reaction of 4-bromophthalan (**1a**) with EDA catalyzed by the free cofactor Ir(Me)-MPIX in NaPi buffer (100 mM, pH 6.0). Without the protein, the free cofactor reaction gave a mixture of products resulting from single and double insertion of the carbene into the (sp^3) C–H bonds of **1a**. Products from a single insertion into the C–H bonds located *meta* and *para* to the bromine atom were observed in a 1:1 ratio (Scheme 2). Selective carbene insertions into (sp^3) C–H bonds catalyzed by transition-metal catalysts typically involve the use of a donor–acceptor carbene precursor.^[13] There are only a few small-molecule catalysts that effect insertions of acceptor-only carbenes into C–H bonds, and they require the substrate as a solvent or in excess (Scheme 1 A).^[12,14] We reasoned that the pre-organization of substrates within the active site of a protein would afford the opportunity to catalyze site-selective intermolecular C–H functionalization using the substrates as the limiting reagents.

To test the ability of engineered Ir(Me) hemoproteins to differentiate nearly identical C–H bonds and to yield products from insertion of a carbene into just one C–H bond, we conducted reactions of 4-bromophthalan **1a** and EDA with previously engineered myoglobin (Myo) mutants. Evaluation of site selectivity from reactions catalyzed by over one-hundred Ir(Me) Myo mutants,^[10b,d] containing mutations over six active-site residues, from clarified lysate and purified protein reaction screening did not uncover any notable site selectivity beyond that of the free cofactor.

On the basis of our previous studies on artificial metalloenzymes generated from artificial cofactors and CYP119, a P450 from the thermophile *Sulfolobus solfataricus*, we investigated whether selectivity could be obtained from this



Scheme 2. Site-selective intermolecular C–H insertion catalyzed by Ir(Me)-MPIX. Yields refers to the formation of all the carbene insertion products and were determined by GC with dodecane as the internal standard.

scaffold, which possesses a more enclosed active site. We previously showed that the melting temperature of CYP119 is higher (69 °C) than that for the analogous construct from the more commonly studied P450-BM3 (45 °C),^[11] and that carbene insertions into C–H bonds do not require the reductase domain contained within BM3.

We initially screened for selectivity with T213G,C317G, a double mutant in which both the size and hydrogen-bonding ability of the amino acid residue at position 213 are reduced and the native axial heme-ligating cysteine at position 317 is eliminated. In contrast to reactions of the free cofactor, the reactions catalyzed by the double mutant CYP119 enzyme gave only product from insertion of a single carbene (Scheme 2). Although the site selectivity from this initial experiment was negligible, the improved chemoselectivity for a single insertion product was clearly imparted by the protein environment.

Directed Evolution of Ir(Me)-CYP119

To create catalysts for site-selective C–H functionalization of phthalan derivatives, we studied the reaction of 4-bromophthalan **1a** with EDA catalyzed by a mutant library of CYP119 proteins.^[11] This library was constructed from the double-mutant scaffold containing the T213G and C317G mutations, with the introduction of additional mutations at

amino acids proximal to the active site (Figure 1). The mutant library was evaluated by screening in 96-well plates. Reactions were conducted by generating Ir(Me)-MPIX CYP119 in cell lysate by reconstituting each apo-protein mutant variant with a stock solution of Ir(Me)-MPIX cofactor, followed by adding stock solutions of the phthalan and carbene precursor catalyst stock solutions to the 96-well plates. The ratios of isomeric products from the insertion process were measured by high-throughput GC sampling from the 96-well plates.

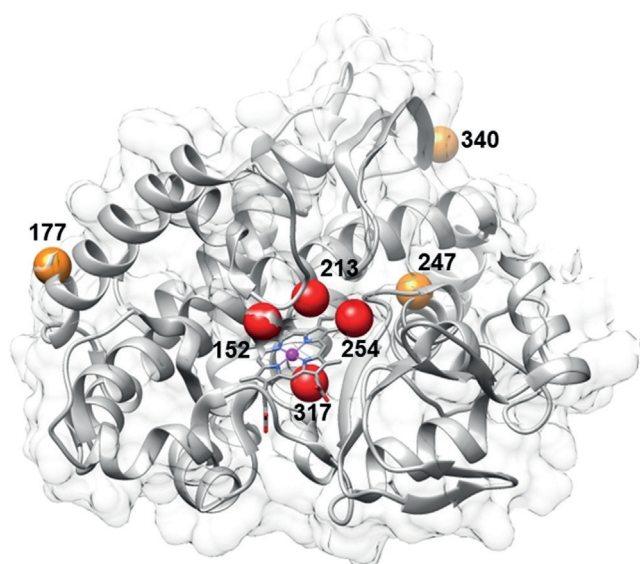


Figure 1. Structure of WT Fe CYP119 (image prepared in Chimera from PDB ID: 1IO7).^[16] Red: mutant positions that were generated by site-directed mutagenesis (A152, T213, V254, C317); orange: additional positions generated by error-prone PCR for *para* selectivity (D177 and H340) and *meta* selectivity (A247).

Results from these experiments indicated that some members of the CYP119 mutant library catalyze reactions at one of the two methylene positions of the substituted phthalan with measurable selectivity. For example, the mutant of CYP119- P_{G1} (*para* generation 1: C317G, T213G, V254L, A152F) preferentially formed the product from insertion into the benzylic C–H bond *para* to the bromine atom over that from insertion *meta* to the bromine atom with a 2.8:1 ratio and 720 turnover numbers (TON). In contrast, the mutant of CYP119- M_{G1} (*meta* generation 1: C317G, T213G, V254A, A152L) preferentially formed the product from insertion into the benzylic C–H bond *meta* to the bromine atom with a 1:2.7 ratio and 690 TON (Scheme 2).

Reactions conducted at catalyst loadings lower than 0.05 mol% led to lower conversion, but they also led to lower site selectivity and gave a mixture of products resulting from a single and double insertion of the carbene into the (sp^3) C–H bonds of **1a**. We assumed the lower chemo- and site selectivity results from an instability of the Ir(Me)-bound CYP119 construct to the reaction conditions, leading to the generation of free cofactor. This decomposition presumably also influences reactions at higher catalyst loadings. Because the proteins at this point of the evolution were generated by mutagenesis only in the active site, we created a new library of

mutants based on P_{G1} and M_{G1} by error-prone PCR that would contain mutations distal to the active site that could stabilize the Ir(Me)-bound P450 construct.

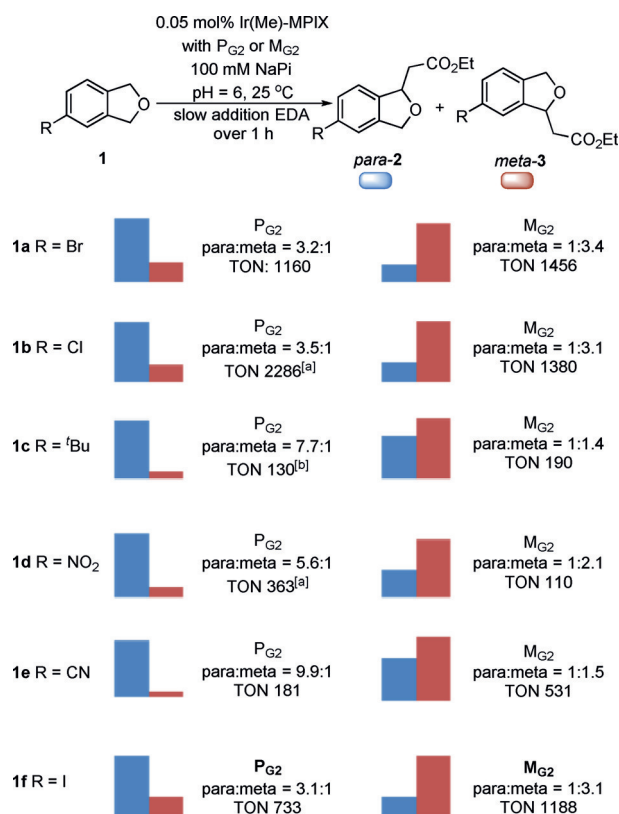
Indeed, three rounds of error-prone PCR led to enzymes that catalyzed the carbene transfer with greater site selectivity, in many cases reaching differences in selectivity greater than 10:1 between pairs of mutants reacting at the two methylene positions of **1a** and with higher turnovers. Eleven mutants were identified that catalyze the reaction to form predominantly the *para* isomer, and 10 mutants were identified that preferentially form the *meta* isomer. These 21 mutants that reacted with selectivity higher than those of the parent mutants were tested for activity across a series of 4-substituted phthalan derivatives, with which small transition-metal complexes,^[12] including the free Ir(Me)-MPIX cofactor, reacted to give insertion products with low selectivity (Scheme S3).

Site-Selective Insertions of Carbenes into Phthalan Derivatives Catalyzed by Ir(Me)-containing CYP119

Two of the 21 mutant variants catalyzed the insertion reaction of EDA into a series of 4-substituted phthalan derivatives containing various functional groups with good TONs and measurable chemo- and site selectivity for either *para* or *meta* isomers. The reaction of 4-bromophthalan **1a** catalyzed by the mutant P_{G2} (P_{G1} + D177N, H340L) gave a 3.2:1 ratio of constitutional isomers in favor of the *para* isomer with 1160 TON, while the same reaction catalyzed by the mutant M_{G2} (M_{G1} + A247S) gave a 1:3.4 ratio of isomers in favor of the *meta* isomer with 1456 TON (Scheme 3). Similar results were observed for reactions of 4-chlorophthalan **1b**. The reaction catalyzed by the mutant P_{G2} formed the product from insertion into the benzylic C–H bond *para* to the chloro-substituent as the major product with a 3.5:1 ratio of constitutional isomers and 2286 TON, while the reaction catalyzed by the mutant M_{G2} yielded the *meta* insertion product in a 1:3.1 ratio and with 1380 TON.

Phthalan **1c** contains a 4-*t*Bu substituent, which is larger than the halides of **1a** and **1b**. However, the site selectivity values of reactions of this sterically distinct substrate with CYP119 mutants were similar to those of reactions of **1a** and **1b**. The product from insertion into the benzylic C–H bond *para* to the *t*Bu substituent was favored by the mutant P_{G2} in a ratio of 7.7:1 and accomplished with 130 TON. Although the selectivity for the *meta* benzylic C–H bond of **1c** from reactions catalyzed by the mutant M_{G2} was lower than that for reaction of **1a** and **1b**, the selectivity, nevertheless, was reversed to 1:1.4 in favor of the *meta* isomer with up to 190 TON. Thus, we were able, again, to identify a pair of mutants (P_{G2} and M_{G2}) that react with nearly a > 10:1 reversal of site selectivity between mutants for the two types of C–H bonds present in **1c**.

Nitro-substituted reactants rarely undergo C–H bond functionalization, and the 4-nitro-substituted phthalan **1d** previously did not react with EDA when catalyzed by a small-molecule iridium species.^[12] However, the reaction of this substrate with EDA catalyzed by an iridium-containing ArMs



Scheme 3. Scope of substrates for which the CYP119 mutants P_{G2} and M_{G2} gave opposite site selectivity. TONs refers to the formation of both *meta* and *para* benzylic C–H bond insertion products and were determined by GC with dodecane as the internal stander. [a] 0.02 mol% Ir(Me)-MPIX CYP119 was used. [b] 0.1 mol% Ir(Me)-MPIX CYP119 was used.

formed the product from insertion into the C–H bond. Although the turnover numbers from this reaction were lower than those from reactions of other substrates, the reaction of 4-nitrophthalan **1d** catalyzed by the mutant P_{G2} formed the product from reaction of the benzylic C–H bond *para* to the nitro substituent as the major isomer with a 5.6:1 ratio over that from reaction at the C–H bond in the methylene group *meta* to the nitro substituent (363 TON). The same reaction catalyzed by the mutant M_{G2} preferentially formed the product from reaction at the methylene C–H bond *meta* to the nitro substituent with a 1:2.1 ratio of constitutional isomers. A similar preference for reaction with the methylene C–H bond *para* to the substituent was observed from the reaction of 4-cyanophthalan **1e** catalyzed by P_{G2} with a 9.9:1 ratio over the reaction at the methylene C–H bond *meta* to the cyano substituent (181 TON). The selectivity was reversed to 1:1.5 in favor of reaction at the benzylic C–H bond *meta* to the cyano substituent for the reaction catalyzed by the mutant M_{G2} (531 TON), again demonstrating a 10:1 reversal of site selectivity for a pair of mutant ArMs. Reaction of the 4-iodophthalan **1f** also occurred with good selectivity for insertion at the *para* position (3.1:1, 733 TON) when catalyzed by the mutant P_{G2}, whereas the same selectivity in favor of reaction at the *meta* position (1:3.1) was observed for the reaction catalyzed by mutant M_{G2} (1188 TON).

Substrates for which the Mutant Enzymes Selectively React at the *para* C–H Bond

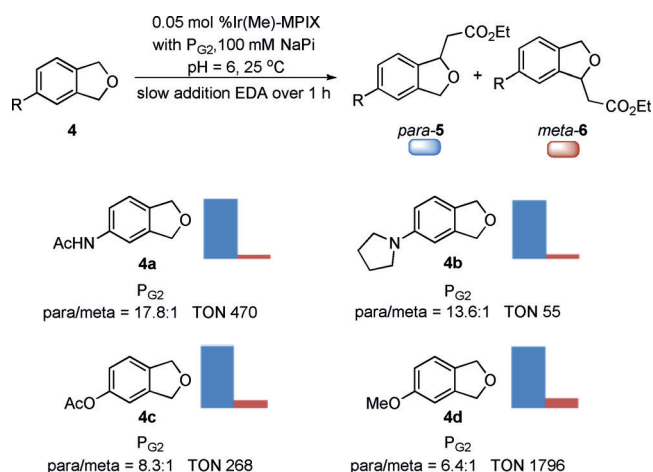
In addition to reacting site-selectively for one C–H bond over another similar C–H bond, the ArMs we report preferentially react with carbene insertion into benzylic C–H bonds over other potential positions. The reaction of amide-substituted phthalan **4a** catalyzed by P_{G2} formed the product from insertion of the carbene into the C–H bond with 17.8:1 selectivity for the benzylic C–H bond *para* to the amide substituent and 470 TON. No product from insertion of the carbene into the N–H bond was observed from reactions catalyzed by any of the Ir(Me)-bound CYP119 constructs.

Likewise, the products of the reactions of EDA with amine-substituted phthalan **4b** catalyzed by the free Ir(Me)-porphyrin and artificial enzyme were distinct. The reaction of EDA with **4b** catalyzed by Ir(Me)-MPIX gave products from single and double carbene insertion into the C–H bond alpha to nitrogen in the pyrrolidine. However, the reaction catalyzed by the mutant P_{G2} occurred with high chemo- and site selectivity for the C–H bond *para* to the amine substituent (13.6:1 *para* selectivity and 55 TON).

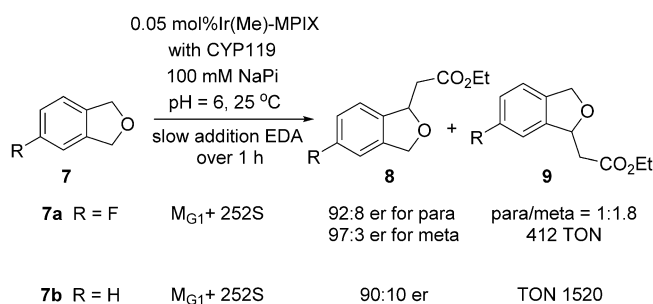
The reactions of EDA with ester-substituted phthalan **4c** and ether-substituted phthalan **4d** catalyzed by P_{G2} also occurred with higher site selectivity compared to the reactions catalyzed by free cofactor. The reaction catalyzed by Ir(Me)-MPIX occurred with low TONs and almost no selectivity for the methylene units *para* and *meta* to the ester- and ether substituents (Scheme S3). However, selectivity of 8.3:1 was observed for reaction at the methylene position *para* to the ester substituent of **4c** when catalyzed by the mutant P_{G2} (268 TON). The reaction of **4d** occurred with 1796 TON and 6.4:1 selectivity for the methylene *para* to the methoxy substituent. These reactions in Scheme 4, combined with the reactions of cyano- and nitro-substituted phthalans in Scheme 3, illustrate the potential of ArMs to enhance *para*-selectivity regardless of the electron-donating properties of the substituents.

Enantioselective Intermolecular C–H Insertion Catalyzed by Ir(Me) CYP119

Our results show that the environment within the ArMs can control the number of insertion events and the site selectivity of the atom-transfer, and it can confer tolerance of several potentially reactive functional groups. We have shown previously that C–H insertion reactions catalyzed by Ir(Me) P450s can occur in an enantioselective fashion.^[11] Because the current work is focused on assessing the ability of ArMs to catalyze reactions with site selectivity, we did not perform extensive evolution to enhance enantioselectivity. Nevertheless, some of the reactions did occur with substantial enantioselectivity when catalyzed by members of the mutant library. For example, the reactions of EDA with 4-fluorophthalan (**7a**) occurred to give the major constitutional isomer in 97:3 er and the minor isomer in 92:8 er when catalyzed by M_{G1} + P252S (Scheme 5). The ratio of constitutional isomers was 1:1.8 in favor of the *meta* isomer. Although this site selectivity is lower than that of reactions of other substituted

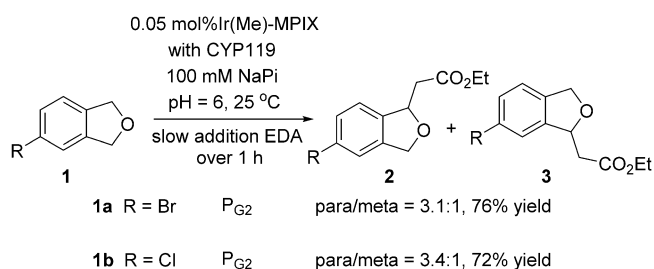


Scheme 4. Scope of substrates for which mutant enzymes are selective for reaction at the C–H bond in the methylene group *para* to the substituent. TONs refer to the formation of products from insertion into the C–H bond of the methylene unit both *meta* and *para* to the substituent and were determined by GC with dodecane as the internal standard.



Scheme 5. Enantioselective intermolecular C–H insertion catalyzed by Ir(Me) CYP119. TONs refer to the formation of products from insertion into the C–H bond of the methylene group both *meta* and *para* to the substituent with dodecane as the internal standard. The er values were determined by HPLC with a chiral column.

phthalans, it does show that the enzyme can react with significant site selectivity and enantioselectively for two C–H bonds that differ only by a remote fluorine versus hydrogen on the aryl ring. A fluorine atom has the closest size to hydrogen of any atom, and has the closest Hammett parameter to hydrogen of nearly any substituent.^[15] In comparison with our prior results from the reaction of phthalan and EDA catalyzed by an Ir(Me) P450 (84:16 er, 324 TON),^[11] higher enantioselectivity and reactivity was observed from the same reaction catalyzed by the mutant $M_{G1} + P252S$ (90:10 er and 1520 TON).^[6a] The feasibility of this reaction on a synthetic scale was assessed by conducting the reactions of **1a** and **1b** on a 0.3 mmol scale (Scheme 6). The yields of isolated product and selectivity were similar to those for reactions on a 3 microgram scale.



Scheme 6. Scale-up the reactions catalyzed by mutant P_{G2} .

Stability of a Series of Ir(Me) P450 Mutants

The majority of the mutations (A152, T213, V254, and C317) were located within the active site; however, some mutations, especially those generated by error-prone PCR (D177, H340, and A247), are located far from the active site in both P_{G2} and M_{G2} (Figure 1). As noted previously in this paper, these additional mutations appear to enhance the stability of the Ir(Me)-bound P450 construct because they have a larger influence on turnover number than on site selectivity (Scheme 3 and 4). To assess this line of reasoning, we evaluated the stability of the Ir(Me)-bound CYP119 construct to temperature and reaction components.

To assist our evaluation of the effect of the mutations distal to the active site, we generated two mutants, M_{max} ($M_{G2} + D177N$, H340L) and P_{max} ($P_{G2} + A247S$), which contain all of the mutations peripheral to the active site in either M_{G2} or P_{G2} . Like P_{G1} , P_{max} catalyzed the reaction of **1a** with a similar site selectivity ratio of 3:1 in favor of the *para* isomer, but the turnover number of the reaction catalyzed by P_{max} (1450 TON) was twice as high as that of the reaction catalyzed by P_{G1} (720 TON; Figure 2). Likewise, the site selectivity of the reaction catalyzed by M_{max} was similar to that of the reaction catalyzed by M_{G1} (1:2.7 for M_{G1} vs. 1:3.5 for M_{max}), but the TON of the reaction catalyzed by M_{max} (1834 TON) was more than three times higher than that of reactions catalyzed by the parent M_{G1} mutant (580 TON) (Figure 2). These results further support the conclusion that the mutations resulting from error-prone PCR (177N, 340L and 247S) stabilize Ir(Me) P450 during the course of the reaction.

Recently, it was reported that modification of the protein scaffold during cyclopropanation of alkenes catalyzed by repurposed heme enzymes and ArMs can occur by carbene transfer into the amino acid residues of the host protein.^[10c, 17a,b] Because Ir(Me)-MPIX is a highly active catalyst for carbene transfers, we suspected that this modification could limit the TON of the Ir(Me)-bound P450 construct and that these distal mutations might be retarding this modification. However, masses corresponding to addition of EDA to the protein or addition of the methyl group of the Ir(Me) cofactor to the protein were not observed from analysis of intact proteins by ESI-MS after addition of EDA to the six mutants of Ir(Me)-PIX CYP119 that gave the highest selectivity or addition of EDA and 4-bromophthalan **1a** to the same six mutants.^[17c] Instead, the carbene undergoes rapid formation of fumarate and maleate (Figure S79).

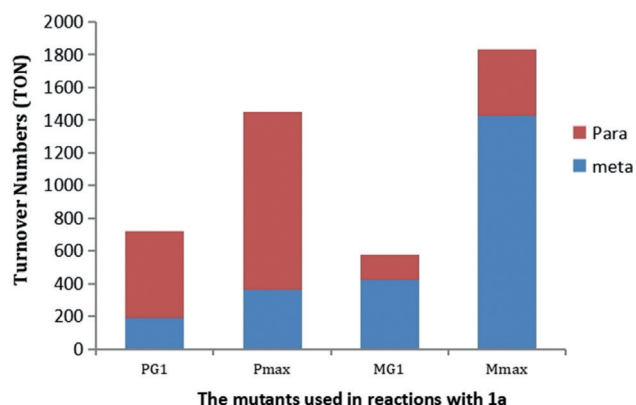


Figure 2. Activity studies of different mutants with slow addition of EDA with **1a**. TONs refers to the formation of both *meta* and *para* C–H bond insertion products with dodecane as the internal standard.

Perhaps the high rate of dimerization prevents reaction of the carbene unit with amino acid residues of the protein scaffold.

Prior studies on repurposed and artificial metalloenzymes for carbene transfer showed that the CD spectrum changed upon exposure to EDA.^[10c,17a] Thus, we measured the CD spectra of P_{G1}, P_{G2}, P_{max}, M_{G1}, M_{G2}, and M_{max} in the apo and in the holo forms of Ir(Me) CYP119 before and after exposure to EDA. The CD spectra are indistinguishable from each other. No notable differences between the CD spectra of the apo- and holo-CYP119 form were observed, or between the CD spectra of these two proteins and that of the WT-CYP119 protein. The α -helicity ($[\theta]_{222}/[\theta]_{208}$) ratio was within 1.00 and 0.96 for all of the Ir(Me)-bound P450 constructs, and these values are similar to those reported for the WT-CYP119 protein (Figures S60–S71).^[18]

The melting temperatures (T_m) of a series of mutants were measured by differential scanning calorimetry. No significant differences among the T_m values of the mutants were observed; the average T_m value was 67.5 °C with a standard deviation of 0.2 °C among all the mutants (Table S2 and Figures S84–S89). The reported T_m value of WT-CYP119 is 91.9 °C. It is not surprising that the T_m values of the mutants of Ir(Me)-MPIX CYP119 are lower than that of WT-CYP119, given the mutations and exchange of an axially ligated Fe-heme cofactor for a non-ligated artificial Ir(Me)-MPIX cofactor.^[19] However, the similarity of the T_m values of the mutants indicate that changes in the active site or in the periphery of the protein have little effect on the folding and thermal stability of the Ir(Me)-PIX CYP119. Therefore, the higher TON of the systems containing peripheral mutations seems to result from greater stability of the Ir(Me)-bound P450 constructs during catalysis.

To probe the stability of CYP119s toward the reaction components (substrate, product, solvent, etc.), Ir(Me)-bound CYP119 mutants were treated with components of the reaction mixture for one hour prior to initiating reactions (Figures S55–S59). This pretreatment of Ir(Me) CYP119s with the reaction products influenced the selectivity of the reaction. For example, reactions of 4-bromophthalan **1a** with EDA conducted after treating P_{G1} or M_{G1} with 4-chloropha-

lan-product **2b** for one hour occurred with negligible site selectivity, whereas the same reaction conducted after treating M_{max} or P_{max} with **2b** for one hour occurred with site-selectivity ratios of 1:2 favoring the *meta* isomer and 1.8:1 favoring the *para* isomer, respectively (Figures S58,59). These results imply that the peripheral mutations stabilize the enzyme toward the C–H insertion products. This trend in effect of product on selectivity is similar to the trend in stability of the protein determined independently by CD (Figures S79–S82). Although difficult to correlate quantitatively, we found that the concentrations of the proteins containing peripheral mutations (P_{max} and M_{max}) were higher than those of the parent mutants P_{G1} and M_{G1} after exposure of equal concentrations of the Ir(Me)-bound P450 proteins to acetate-substituted product **6c**.^[20]

Conclusion

In summary, artificial metalloenzymes containing a noble-metal porphyrin (Ir(Me) CYP119) are capable of catalyzing the intermolecular insertion of acceptor-only carbenes into nearly equivalent C–H bonds with up to 17.8:1 site selectivity for a series of 4-substituted phthalans. Through laboratory evolution, a nearly 10:1 ratio between either the *meta* or *para* constitutional isomers was obtained using two distinct Ir(Me)-bound CYP119 mutants. Although not the primary focus of this study, enantioselectivity of 97:3 can be obtained with the current mutant library for the insertion of EDA into 4-fluorophthalan. The insertion reactions catalyzed by the Ir(Me) P450s tolerated a series of functional groups that have not been tolerated by small-molecule catalysts for analogous insertions of acceptor-only carbenes. Furthermore, unlike other ArMs or repurposed heme enzymes, the Ir(Me) P450s are less susceptible to deactivation from EDA insertion into the protein framework. In general, this work highlights how the combination of a highly active transition-metal active site with a readily-evolvable protein framework can lead to regioselectivities for abiotic reactions that would be difficult to achieve with small-molecule catalysts.

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